MICROBAC®

Microbac Protocol

Testing Pre-Saturated or Impregnated Towelettes for Tuberculocidal Effectiveness

Mycobacterium bovis (BCG)

Testing Facility
Microbac Laboratories, Inc.
105 Carpenter Drive
Sterling, VA 20164

Prepared for
STERIS CORPORATION
7405 Page Avenue
St. Louis, MO 63133-1032

February 10, 2017

Microbac Protocol No.: 429.1a.02.10.17

Microbac Project No.: 429 - 350

OBJECTIVE:

This test is designed to substantiate tuberculocidal effectiveness for impregnated or pre-saturated towelettes, single or multiple uses, to be registered with the Environmental Protection Agency and Health Canada. The test incorporates appropriate aspects of AOAC method 965.12, Tuberculocidal Activity of Disinfectants (2012). The test evaluates the effectiveness of products as disinfectants for contaminated surfaces. The test follows the "Germicidal Spray Products as Disinfectants" test as described in the Official Methods of Analysis, Eighteenth Edition, 2012, AOAC and the EPA Notice of Efficacy Requirements for Pre-Saturated or Impregnated Towelettes for Hard Surface Disinfection. This test also meets the EPA OCSPP 810.2000 and 810.2200 Product Performance Test Guidelines as applicable and follows the US EPA OPP Microbiology Laboratory SOP for Disinfectant Towelette Test against Mycobacterium bovis (BCG), SOP Number: MB-23-02, Date Revised: 03-05-13, where appropriate.

TESTING CONDITIONS:

Using a single test substance, ten carriers, per lot will be tested using two lots.

The carriers, inoculated with *Mycobacterium bovis* (BCG), will be wiped for a specified time directed by the sponsor or label instruction and held for the exposure time and at the temperature specified by the sponsor. The carriers will be cultured, incubated and observed for visible growth.

MATERIALS:

A. Test, control and reference substances will be supplied by the sponsor of the study (see last page).

- The identity, strength, purity, and composition, or other characteristics which will appropriately define the test, control, or reference substance shall be determined for each batch and shall be documented by the sponsor before its use in a study. Methods of synthesis, fabrication, or derivation of the test, control, or reference substance shall be documented and retained by the sponsor.

- When relevant to the conduct of the study the solubility of each test, control, or reference substance shall be determined by the sponsor before

the experimental start date. The stability of the test, control, or reference substance shall be determined by the sponsor before the experimental start date or concomitantly according to written standard operating procedures, which provide for periodic analysis of each batch.

The test substance will be tested as supplied by the sponsor unless directed otherwise. All operations performed on the test substance such as dilution or specialized storage conditions must be specified by the sponsor before initiation of testing.

The sponsor assure Microbac testing facility management that the test substance has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

Microbac will retain all unused test substances after completion of the test for one year, and will only discard them with client permission in a manner that meets the approval of the safety officer.

Materials supplied by MicroBioTest including but not limited to:

1. Challenge organism, required by AOAC:
   Mycobacterium bovis (BCG). OrganonTeknika, Corp.

2. Media and reagents:
   a. 0.85% NaCl containing 0.1% Polysorbate 80 (SS+)
   b. Middlebrook 7H11 agar
   c. Kirchner's medium
   d. Middlebrook 7H9 broth
   e. Phosphate Buffered Dilution Water (PBDW)
   f. Modified Proskauer-Beck Medium (MPB)
   g. Heat-inactivated (HI) fetal bovine serum (FBS), if required.
   h. Neutralizer: Heat-inactivated Horse Serum or other suitable solution.

3. Laboratory equipment and supplies, including glass microscope slides (1" x 3" with a 1" x 1" surface for contamination and treatment)

TEST SYSTEM IDENTIFICATION:

All test and control tube racks will be labeled with microorganism, test substance (if applicable) and project number prior to initiation of the study and during incubation. Petri dishes will be labeled with microorganism prior to initiation of the study and microorganism and project number during incubation.

EXPERIMENTAL DESIGN:

A. Preparation of inoculum:

From stock culture, tubes containing 20 mL of MPB will be inoculated by transferring one to two 1 µL loopfuls from stock slants (7H11 agar slant) and incubated in a slanted position at 36±1C while remaining quiescent for 21±2 days.

Using a transfer loop, sufficient growth will be transferred from the surface of the 20 mL culture into a sterile tissue grinder. One mL of SS+ will be added and the culture will be macerated to break up large clumps. Nine mL of MPB will be added to the homogenized culture. The homogenized suspension will be transferred to a sterile tube and the culture will be allowed to settle for 10-15 minutes. The upper portion of the culture will be removed and transferred to a sterile flask and swirl to mix, leaving behind any debris or clumps.

The suspension will be diluted with MPB to achieve 20±1% *T* using a spectrophotometer at 650 nm. The suspension must result in carrier counts of 4 – 6 Log_{10} CFU/carrier.

If requested by the sponsor, HI FBS will be added to the culture to achieve 5% organic load.

B. Preparation of carriers:

The new carriers will be visually screened and discarded if visibly damaged (scratched, chipped or nicked). The carriers will be rinsed with 95% ethanol followed by a rinse with deionized water to remove oil and film on the slides. The carriers will be sterilized by placing them in evaporating dishes matted with two pieces of filter paper, heating them in a hot air oven for two hours at 180C, cooling and storing them at room temperature until use.

C. Carrier inoculation:

Using a positive displacement pipette, a 0.01 mL (10 µL) aliquot of each culture will be transferred onto a one-square inch area on the sterile carriers (in Petri dishes) and immediately spread uniformly over the entire area with a sterile loop. Each dish will be covered promptly and the operation will be repeated for the rest of the carriers. Carriers will be dried for 30±2 minutes at 36±1C. Inoculated carriers will be used for testing within two hours of drying.

Note: The temperature and humidity level of the incubator during the drying of carriers will be monitored and reported.

D. Test substance preparation:

Immediately before testing, pressure will be applied to pouch containing the wipes and liquid in order to rupture the bladder and saturate the wipes. Each canister of test substance will be allowed to equilibrate to room temperature for a minimum of 10 minutes. The pouches containing the towelettes will be inverted or mixed to re-saturate the towelettes.

E. Test:

Note: The temperature and humidity level of the laboratory during the test phase will be monitored and reported.

Ten carriers per lot will be treated based on the following procedures requested by the sponsor:

When using towelettes from a new pouch, the pouch will be agitated to ensure saturation. Do not remove any wipes before use in testing.

Initially, the towelette will be folded lengthwise twice and then folded five times inward beginning from the far end. Then the outside edges will be pulled upward to form a "U" shape and grasped preferably on one side with the thumb and on the other side with the index and middle finger. The folded towelette will be rotated 90°.

Each carrier will be wiped back and forth three times lengthwise with the towelette for a total of six passes across the inoculum. Wiping will be performed within ± 5 seconds. The lid of the Petri dish will be closed. The wiped carriers will be maintained in a horizontal position.

The used end of the wipe will be flipped upward towards self, reoriented appropriately and then used to wipe the next carrier. The next three carriers will be wiped in a similar fashion - the used portion will be folded up-and-over each time.

Once five carriers have been wiped, the towelette will be unraveled. The second lengthwise fold will be unfolded and refolded in the opposite direction. The towelette will be refolded five times as before and the above procedure for wiping the first five carriers will be repeated for wiping the last five carriers.

The contact time will be initiated once the carrier is wiped with the towelette.

Once treated, each carrier will be held in a horizontal position for the exposure time as specified by the sponsor. After the contact period, each carrier will be transferred to tubes containing 20 mL of the Neutralizer using sterile forceps within the ±5 sec. (or ±3 sec.) time limit and shaken thoroughly. For products with ≤1 minute contact time, the transfer will be made within ±3 seconds. The slide can touch both the interior sides of the Petri dish and the neutralizer tube during the transfer, but care will be taken to avoid this contact as much as possible.

Each tube containing the carrier in neutralizer will be thoroughly shaken and the carrier will be transferred to a tube containing 20 mL of MPB broth within 5-10 minutes. Sterilize forceps will be used for each carrier transfer. From each tube of neutralizer, two mL will be subcultured to a tube containing 20 mL 7H9 broth and 2 mL will be subcultured to a tube containing 20 mL Kirchner's medium. Each subculture tube will be shaken thoroughly and the sequence will be repeated for all carriers within 30±5 minutes.

F. Controls:

1. Viability controls:

One contaminated carrier each will be added to tubes containing 20 mL MPB, 20 mL 7H9 and 20 mL KM. The tubes will be incubated with the test to confirm the ability of the recovery media to support growth of the challenge microorganism.

2. Neutralizer effectiveness:

The standardized culture will be serially diluted 10-fold with MPB out to the 10^{-6}(e.g., dilution tubes 10^{-3} through 10^{-6}). 100 µL aliquots of dilutions 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} will be added per tube to demonstrate the recovery of a low level (≤100 CFU/tube) of the test organism in the subculture media.

For each lot, a single carrier per test culture dilution (e.g., dilution tubes 10^{-3} through 10^{-6}) will be exposed to the test substance and processed in the same manner as the test carriers. To each tube of 20 mL 7H9, 20 mL KM and 20 mL MPB media, ≤100 CFU will be added to each tube. All tubes will be incubated alongside the test. When inoculated with ≤100CFU/tube, all treatment tubes must demonstrate growth to have a valid neutralization effectiveness test.

A single sterile carrier per test culture dilution (e.g., dilution tubes 10^{-3} through 10^{-6}) will also be evaluated by omitting test substance treatment followed by processing in the same manner as the test carriers. To each tube of 20 mL 7H9, 20 mL KM and 20 mL MPB media, ≤100 CFU will be added to each tube. All tubes will be incubated alongside the test. When inoculated with ≤100 CFU/tube, the MPB tube with the carrier must demonstrate growth to have a valid neutralization effectiveness test.

Untreated tubes of each media per test culture dilution will also be inoculated with ≤100 CFU for comparison. All tubes will be incubated alongside the test. The untreated carrier tubes should exhibit growth for all media. When inoculated with ≤100 CFU/tube, all media tubes must demonstrate growth to have a valid neutralization effectiveness test.
The concentration of the bacterial suspension inoculated into these tubes will be confirmed by plating100 µL aliquots of appropriate dilutions in

duplicate on 7H11 using spread plating. The plates will be incubated for 17 – 21 days at 36±1C.

3. Sterility controls:

One sterile, uninoculated carrier will be placed into a tube of MPB broth. In addition, 1 tube of each subculture medium with 2 mL sterile neutralizer will be incubated for quality control purposes. Each tube will be shaken thoroughly and all the tubes will be incubated with the efficacy test. Duplicate 7H11 agar plates will also be incubated with the test.

4. Carrier counts:

Three inoculated dried carriers will be randomly selected for evaluation. One carrier will be assayed immediately prior to conducting the efficacy test and two carriers following the test. Each of three carriers will be placed in independent sterile 50 mL polypropylene conical tubes containing 20 mL of MPB and subjected to vortex-mixing for 15 seconds.

After vortex-mixing, 10-fold serial dilutions will be conducted in PBDW. 100 µL aliquots of appropriate dilutions will be plated in duplicate on M7H11 agar using spread plating. Dilutions 10^{-1} through 10^{-3} should produce plates with CFU in the appropriate range. Plates must be dry prior to incubation. All dilutions and plating will be performed within 2 hours of vortexing. The plates will be incubated for 17-21 days at 36±1C.

5. Performance assessment of Media:

The standardized culture will be serially diluted 10-fold out to the 10^{-6}. For solid media, 0.1 mL aliquots from the 10^{-3} to the 10^{-6} dilutions will be spread plated on 7H11 agar plates in duplicate. The plates will be incubated for 17-21 days at 36±1C. Plate counts of 30-300 CFU/plate should result from at least one of the dilutions plated.

For liquid media, each tube of liquid medium (MPB, 7H9, and KM) will be inoculated with 0.1 mL aliquots from the 10^{-3} to the 10^{-6} dilutions in duplicate. Tubes will be incubated for 60 days at 36±1C. At least 1 of the 2 tubes in a set that received a low level of inoculum should produce growth.

G. Incubation:

Unless otherwise indicated, all test tubes used for secondary transfers (MPB, 7H9, and KM) and all controls tubes will be incubated for 60 days at 36±1C and the results will be reported as growth or no growth. If no test culture shows visible growth, the test will be incubated an additional 30 days before the final reading is made. All plates will be incubated for 17-21 days at 36±1C, the colonies will be counted and the average CFU calculated.

H. Confirmation of challenge microorganism:

On the day of the final reading, acid-fast stains will be performed for all test culture tubes demonstrating visible growth and the viability control tubes to verify the presence of the challenge microorganism. In addition, the culture morphology will be observed.

As a secondary verification, isolation streaks from the viabilities and one randomly selected positive tube (if applicable) will be performed on 7H9 or 7H11 agar and incubate for 17-21 days at 36 ± 1C. Following the 17-21 day incubation period, the plates will be evaluated for colony morphology and acid fast stained.

PRODUCT EVALUATION CRITERIA:

The test substance meets effectiveness requirements if no visible growth occurs in any replicate tube, for any of the subculture broths.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance. There are no proposed statistical methods for this test.

- The geometric mean of the carrier counts must be at least 4 – 6 Log_{10} colony-forming units (CFU)/carrier. The log_{10} density (LD) for each carrier will be determined based on the following:
  - Dilutions yielding counts up to 300 CFU will be used.
  - Plate counts of 0 will be included in the calculations.
  - The CFU/mL (of broth) will be calculated:

$$\text{CFU/mL} = \frac{(\text{avg.CFU for } 10^{-x}) + (\text{avg.CFU for } 10^{-y}) + (\text{avg.CFU for } 10^{-z})}{10^{-x} + 10^{-y} + 10^{-z}}$$

  - The CFU/carrier will be calculated by multiplying the CFU/mL by the volume of broth into which the bacteria were harvested from the carrier by vortexing (20 mL).
  - The LD for each carrier will be calculated by taking the Log_{10} of the density (per carrier).
  - The mean LD across carriers is the mean Test LD for the test. The mean Test LD must be at least 4.0 (corresponding to a geometric mean density of 1.0×10^4) and not above 6.0 (corresponding to a geometric mean density of 1.0×10^6).
  - Test LD that are below 4.0 log CFU/carrier or above 6.0 log CFU/carrier invalidates the test.
- Following inoculation of $\leq$100 CFU per tube/plate, growth must be observed for the media evaluated in the performance assessment of Media section.
- The sterility controls must show no growth.
- The viability controls must show growth in all media.
- The neutralization confirmation tubes must show growth following inoculation with $\leq$100 CFU per tube to confirm effective neutralization.

DATA PRESENTATION:

The final report will include the following information:

- The number of positive carriers.
- The average colony-forming units per carrier.
- The results of all controls.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request. This study will be conducted at Microbac, 105 Carpenter Drive, Sterling, VA 20164.

CONFIDENTIALITY:

All data generated at Microbac are held in strictest confidence and are available only to the sponsor and the sponsor designated authorities (if applicable). In turn, no reference to Microbac's promotion of the evaluated test articles may be made public by the sponsor.

REPORT FORMAT:

Microbac employs a standard report format for each test design. Each final report provides the following information:

- Sponsor identification and test substance identification
- Type of test and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results
- Methods and evaluation criteria
- Signed Quality Assurance and Compliance Statements (if applicable)

REGULATORY COMPLIANCE AND QUALITY ASSURANCE (applicable to GLP studies only)

This study will be performed in compliance with the US Environmental Protection Agency's Good Laboratory Practices regulations, 40 CFR 160. Note: information on the identity, strength, purity, stability, uniformity, and dose solution analysis of the test substance resides with the sponsor of the study unless otherwise stated.

The Quality Assurance Unit of Microbac will inspect the conduct of the study for GLP compliance. The dates of the inspections and the dates that findings are reported to the study management and study director will be included in the final report.

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test substance records, final report, and correspondence between Microbac and the sponsor will be stored in the archives at Microbac, 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test substance; challenge microorganism used; media and reagent identification; and the type of neutralizers employed in the test will be addressed in a project sheet issued separately for each study. The date the study director signs the protocol will be the initiation date. All project sheets will be forwarded to the study sponsor.

MISCELLANEOUS INFORMATION: The following information is to be completed by the sponsor prior to initiation of the study (please check all applicable open boxes):

A. Name and address: STERIS CORPORATION
7405 Page Avenue
St. Louis, MO 63133-1032

B. Test substance information:

Test substance name	EXP 16042	
Lot No.	Lot No. 1	Lot No. 2
	PFR2566A	PFR2566B
Active ingredient(s)	Hydrogen Peroxide and Peracetic Acid	

C. Test Conditions:

Dilution	Ready to Use		
Diluent	Not applicable		
Lower Certified Limit (LCL)	■ yes	☐ No	☐ Not applicable
Contact time	9.5 minutes		
Contact temperature	Ambient Room Temperature (20±1C)		

D. Organic load – serum (HI FBS) added to achieve 5% in the inoculum: ■ yes ☐ no

E. Precautions/storage conditions: MSDS and/or C of A provided: ■ yes ☐ no

REPORT HANDLING AND STUDY CONDUCT: EPA & Health Canada, GLP

PROTOCOL APPROVAL:

Sponsor Signature: _____ Date: 2/16/17
DAN KLEIN, STERIS CORPORATION

Study Director Signature: _____ Date: 2/17/17
Travis R. Farley, Microbac